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Formation of *N*-(2-Hydroxyethyl)valine Due to Exposure to Ethylene Oxide via Tobacco Smoke: A Risk Factor for Onset of Cancer[1]

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Human exposure to ethylene oxide (EtO) occurs mainly through inhalation of occupational polluted air and tobacco smoke. EtO is able to react with DNA and proteins producing some molecular adducts. One of these, resulting from reaction between EtO and valine in hemoglobin, is *N*-(2-hydroxyethyl) valine (HOEtVal). This adduct represents a biological effective dose marker, the level of which correlates linearly with the alkylating activity occurring in DNA. The aim of the present study was to measure HOEtVal in 146 urbanized adult and healthy subjects, nonoccupationally exposed to EtO, and to correlate it with smoke habits. HOEtVal showed a direct positive relationship to tobacco smoke exposure quantified by questionnaire, urinary cotinine ($r = 0.64509$), and the number of cigarettes ($r = 0.6308$) actively or passively smoked. Results relative to HOEtVal and urinary cotinine in adults distinguish well between active and passive smokers but do not allow distinguishment between passive smokers and nonsmokers. Nevertheless, several authors demonstrated a very good capacity of cotinine to discriminate inside groups of adolescents passive smokers. Therefore, the future objective of the present study is a closer inspection of the two biomarkers with respect to passive exposure to tobacco smoke considering a large group of adolescents. Finally, the correlation between urinary cotinine and HOEtVal increases knowledge about early steps of the carcinogenic process due to active exposure to tobacco smoke. © 1999 Academic Press

Key Words: **tobacco smoke; hydroxyethylvaline; cotinine; molecular adduct; public health.**

[1]This study was conducted in accordance with national and institutional guidelines for the protection of human subjects.

INTRODUCTION

The major use of ethylene oxide (EtO) is as an intermediate compound in the production of various chemicals; a small fraction of the total consumption is used for the fumigation and sterilization of foodstuffs and medical equipment (WHO, 1985).

Human exposure to this alkylating agent occurs mainly through inhalation of occupational polluted air (Osterman-Golkar and Bergmark, 1988; Ribeiro *et al.*, 1994) and the gaseous phase of tobacco smoke (Guerin, 1987). EtO can also be present in the human body as a consequence of metabolic activation of ethylene (Segerbäck, 1983), the latter being both an urban pollutant (Sawada and Totsuka, 1986) and a component of the gaseous phase of tobacco smoke (Guerin, 1987).

Even if the prevention systems recently adopted have induced a reduction of human exposure to EtO, it should be remembered that ethylene oxide is considered by IARC (IARC, 1994) carcinogenic to humans (group 1). The epidemiological studies reported on humans occupationally exposed to ethylene oxide evidenced that this epoxide causes an excess of leukemia and probably of solid tumors. Low but chronic levels of human exposure must also be considered as a potential risk factor for tumor formations.

EtO is an electrophilic compound able to react with DNA [illegible] activation. The reactivity of this gas toward different nucleophilic sites in macromolecules has been characterized (Osterman-Golkar *et al.*, 1983; Segerbäck, 1990). The major reaction product in DNA is *N*-7(2-hydroxyethyl)guanine, while the major reactive sites in the four peptidic chains of human

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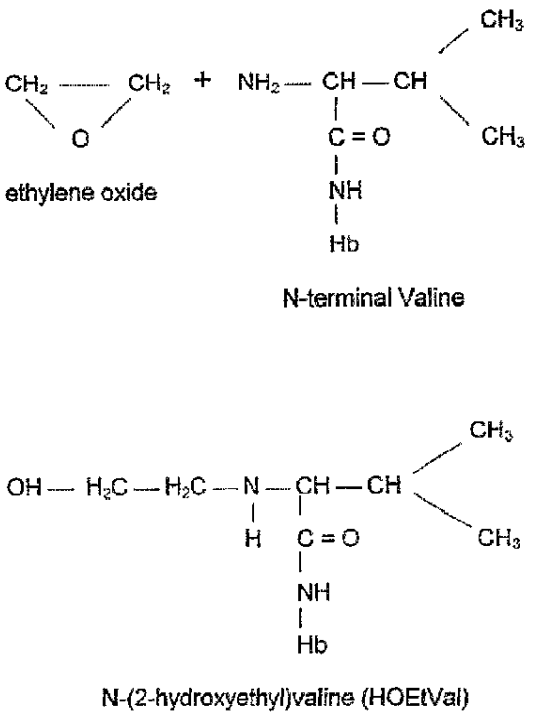

FIG. 1. The formation of *N*-(2-hydroxyethyl) valine (HOEtVal).

hemoglobin are cysteine, histidine, and, in particular, N-terminal valine (Bryant and Osterman-Golkar 1991).

The molecular adduct resulting from reaction with valine (Fig. 1), namely *N*-(2-hydroxyethyl)valine (HOEtVal), represents a biological effective dose marker related either to the assumed dose of ethylene oxide or to the assumption of ethylene, which is metabolized to ethylene oxide. The level of this molecular adduct correlates linearly with the alkylating activity occurring in DNA. With respect to this linear relationship, Farmer calculated that 10 pmol of HOEtVal/g of globin corresponds to 0.33 pmol of adduct/g of DNA (Farmer *et al.*, 1987). Therefore, findings of the distributions in humans of hydroxyethylvaline should be considered an indication of potential cancer risk and its level can be used for estimating the risk in different population groups. Furthermore, the use of hemoglobin as a test molecule for capturing reactive species that give rise to chemically stable adducts allows the integration of the exposure over the lifespan of erythrocytes (4 months in humans) (Kautianen and Törnqvist, 1991).

The aim of the present study was to measure HOEtVal formation in the hemoglobin of 146 urbanized adult and healthy subjects, nonoccupationally exposed to EtO, and to correlate it with smoke habits, the latter being the major source of environmental exposure to EtO. To gain information on smoke habits, a questionnaire was used as a subjective marker and urinary cotinine, a well-known objective internal dose marker of exposure to tobacco smoke, was also used (Bono *et al.*, 1996).

The results allow the validation of the analytical method for the detection of this biologically effective dose marker and the evaluation of its distribution inside the selected population. Furthermore, the correlation between urinary cotinine and HOEtVal increases the knowledge about early steps of the carcinogenic process due to active exposure to tobacco smoke.

MATERIALS AND METHODS

A. Choice of Subjects

One hundred forty-six subjects ($\geq$18 years old) were randomly chosen among AVIS donors (Italian Association of Blood Donors) of Turin city, northwestern Italy, from December 1995 to February 1996. The only selection process adopted was the involvement of an equal number of males and females. The procedure for each subject entailed administration of a questionnaire and collection of a venous blood sample and a urine sample.

B. Data Collection and Analysis

1. Questionnaire

One interviewer administered a questionnaire obtaining information about: (a) individual characteristics (sex, age, residence, smoking habits), (b) family (number of components, smoking habits of each of them), (c) professional activity.

2. Blood Samples

From each subject 5 ml of venous blood was collected in heparinized tubes. The procedure for HOEtVal analysis is summarized in Fig. 2.

2a. Isolation of globin The collected blood was centrifuged (2500*g*, 10 min) and washed three times using NaCl (0.9%). In order to lyse the erythrocytes, 10 ml of ultrapure water was added. At this point, storage of samples at -20°C for 5 months proved not to form any artifact (Törnqvist, 1990). The emolysate was centrifuged (20000*g*, 10 min) and dialysis in water of the supernatant (kept in the dark at $+4^{\circ}$C) was performed. A solution of 2-propanol,

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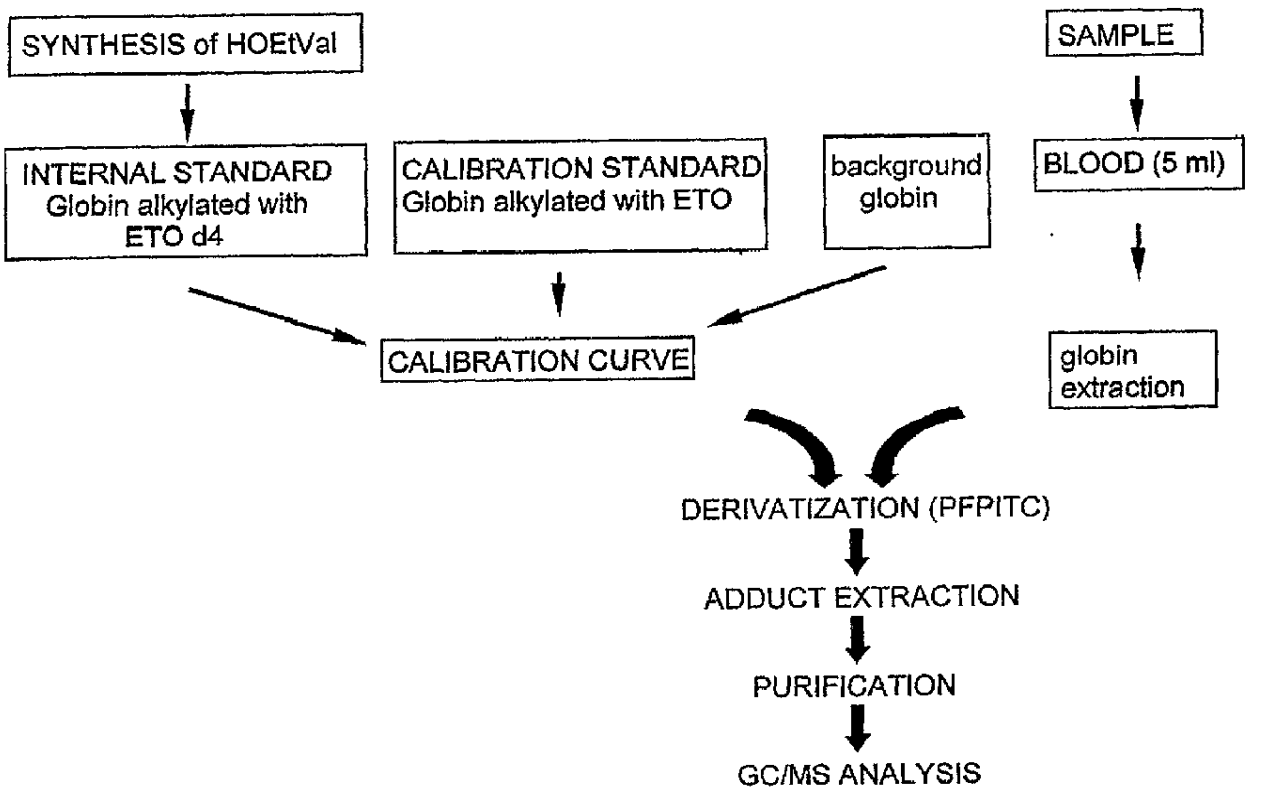

FIG. 2. Procedure for HOEtVal analysis.

acidified with HCl (0.5%), was added (1:6) to the dialysis product. Isolation of globin was obtained by further addition of 4 ml of ethyl acetate, centrifugation (5000*g*, 10 min), and precipitate washing using ethyl acetate and pentane (1:1). Lastly, the precipitate was dried and stored at −20° C) (Törnqvist 1994). At this temperature, qualitative or quantitative alterations of hemoglobin are prevented for up to 4 years (Törnqvist, 1990).

2b. Derivatization of globin. This procedure consists of the application of Edman degradation method modified by Törnqvist (Törnqvist *et al.*, 1986). Fifty milligrams globin was weighted, diluted in formamide (1.5 ml), and buffered to pH 6.5–6.8. In each sample 50 µg of internal standard (globin alkylated with EtO $^2d_4$) was added and the sample was shaken until dissolution. Details concerning the internal standard are discussed below. Subsequently 7 µl of pentafluorophenyl isothiocyanate (PFPITC) was added as derivatizing agent and then the sample was shaken overnight at room temperature, put into a water bath (45° C for 90 min), and extracted using diethyl ether. The extract was dried by a stream of nitrogen and the residue was dissolved in 1 ml of toluene and washed using ultrapure water, NA_2CO_3, 0.1 M, and ultrapure water again. In this manner the *N*-(2-hydroxyethyl)valine-pentafluorophenyl thiohydantoin (HOEtVal-PFPTH) was isolated by evaporation under nitrogen at 60° C.

2c. Purification of samples. Each sample was purified by means of a C-18 cartridge (Törnqvist, 1991) (before the GC/Ms analysis) in order to eliminate most impurities.

2d. Calibration curve. The calibration curve was prepared according to the following steps: (1) synthesis of HOEtVal (not commercially available), (2) preparation (alkylation *in vitro*) of calibration and internal standards, (3) determination of the alkylation degree obtained, (4) preparation of the calibration curve.

(1) 7.5 g of 2-bromoisovaleric acid and 20 g of ethanolamine were reacted in 5 ml of ultrapure water for 15 h at warm temperature. The reaction product was cooled at room temperature and then

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was precipitated by addition of acetone (250 ml). The precipitate was filtered and dried. Subsequently, the product was diluted in 25 ml of ultrapure water and crystallized using 70 ml of a solution of acetone–ethanol (2 : 1 v/v) (Törnqvist, 1994). The purity of HOEtVal was determinated by means of fast atom bombardment (FAB)-mass spectrometry, the details of which are discussed below.

(2) Two types of standards were used. The *internal standard* was prepared *in vitro*, by alkylating 5 ml of erythrocytes with EtO^2d_4; this standard allows the quantification of HOEtVal in samples. The *calibration standard* was also prepared *in vitro*, but by alkylating 5 ml of erythrocytes with EtO; this standard is used to verify the linearity of response and reproducibility of analysis.

Both standards were prepared as follows: 5 ml of erythrocytes (without serum), suspended in a phosphate buffer solution (PBS) 0.01 M, pH 7.4, was added with a cold physiological solution (0.9%) until a final volume of 25 ml was reached; 40 mM($\approx$1 ml) EtO^2d_4 and EtO were respectively added to obtain the internal and calibration standards. These two mixtures were stored at 37° C for 24 h. The reaction was completed by washing the erythrocytes with a physiological solution (NaCl 0.9%). Isolation of the two globins is executed according to procedure 2a.

(3) To determine the alkylation degree of the internal standard, a mixture of 10 mg of globin alkylated with EtO^2d_4 and 20 nmol of HOEtVal previously synthesized was prepared. This mixture was hydrolyzed using 2 ml of 6 M HCl at 120° C overnight. Subsequently, the remaining solution was evaporated and the hydrolyzed product was dissolved in 6 ml of a solution of 1-propanol and $KHCO_3$, 0.5 *M* (1 : 2 v/v). The pH was regulated to 8.6. One half of the hydrolysate was derivatized with 10 µl of PFPITC and treated as previously described (derivatization of globin and purification of samples).

At the same time a mixture of the two standard globins (10 mg of both) was treated identically, as described for the mixture of HOEtVal-globin alkylated with EtO^2d_4. This second procedure allows the determination of the amount of molecular adduct (the alkylation degree) in the calibration standard. Analysis of the alkylation degree of internal and calibration standards was performed by means of GC/MS (Törnqvist, 1994).

The calibration curve was prepared using aliquots of 50 mg of a background globin, having a low level of HOEtVal (i.e., a globin of a young nonsmoker subject), dissolved in 1.5 ml of formamide, and different quantities of calibration and internal standards. These solutions were prepared as follows.

Calibration standard solution: Calibration standard (250 µg) and 25 mg of background globin as carrier globin were added to 5 ml of formamide. From this solution 2.5, 5, 25, 50, 100, 200, 300, 500 µl were transferred into clean vials to prepare the calibration curve solutions.

Internal standard solution: Two milligrams of internal standard and 25 mg of carrier globin were added to 5 ml of formamide. From this solution 50 µl was withdrawn and added in each sample and in each point of the calibration curve. After this addition, the preparation of the calibration curve continued by addition 7 µl of PFPITC, as described above (Törnqvist *et al.*, 1989).

2e. Determination of purity of the synthesized HOEtVal, FAB-MS analysis. A few particles of the synthesized *N*-(2-hydroxyethyl)valine were dissolved in two drops of glycerol. About 2 µl of the resulting solution was loaded onto a FAB probe and positioned in the mass spectrometer ion source, to be exposed to ion bombardment. An Ion-Tech, Ltd., ion gun was utilized to produce a beam of Cs^+ ions accelerated at 27 keV. These ions hit the surface of the solution inducing the sputtering of superficial layers and transfer of neutral and ionic-sputtered species into the gas phase. Ionic species were accelerated to either +5000 eV or −5000 eV and mass-analyzed, yielding positive ion and negative ion FAB mass spectra. The ion source was maintained at room temperature. The mass-analyzer was continuously scanned from m/z 60 to m/z 600 in 8-s cycles, at m/Δm 1000 resolution.

Positive and negative ion FAB mass spectra showed respectively the protonated ($[MH]^+$, m/z 162) and deprotonated ($[M-H]^-$, m/z 160) *N*-(2-hydroxyethyl)-valine molecular ion as the base peak. A second peak in the spectra was likely to correspond to the unreacted valine ($[MH]^+$, m/z 116 and $[M-H]^-$, m/z 114) and accounted for some 3%÷6%. All the remaining peaks corresponded to either glycerol clusters or adducts between the product and glycerol. No further reaction by-product was evident in the spectra.

2f. GC/MS analysis. Dried samples were dissolved in 50 µl of toluene and immediately analyzed. GC/MS analysis was performed using a Finnigan-MAT 95 Q (Bremen, Germany) mass spectrometer in the electron capture (negative chemical ionization) mode. The instrument carries three analyzers in series, magnetic, electrostatic, and quadrupole, but, in the present experiments, ions were detected at the first dynode-electron multiplier device, which is located immediately after the electrostatic sector.

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A Varian 3400 (Palo Alto, CA) gas chromatograph was interfaced to the mass spectrometer. Samples (1 μl) were introduced in the split/splitless injector of the GC, kept at 300°C and working in the splitless mode for 60 s. The sample mixture was separated on a bonded-phase DB-5-MS capillary column (J&W Scientific, Folsom, CA), 30 m long, 0.25-mm internal diameter, and 0.25-μm film thickness. The oven temperature was programmed as follows: isothermal at 100°C for 1 min, from 100 to 190°C at 20°C/min, then from 190 to 235°C at 4.5°C/min, and again from 235 to 300°C at 20°C/min, isothermal at 300°C for 10 min. The column end was introduced directly into the ion source of the mass spectrometer, through a transfer line heated to 270°C.

Ionization of the analytes eluted by the GC column was achieved by electron capture chemical ionization (EC). Methane was introduced into the ion source as a moderating gas at an approximate pressure of 50 Pa (0.5 mBar). The electron emitting filament was set to 200 V and the electron current at 0.2 mA. The ion source temperature was maintained at 190°C.

The magnetic analyzer was either continuously scanned from m/z 310 to 380 with fast cycling times (0.5÷0.8 s) or run under selected ion monitoring conditions, using the ions at m/z 348 and 352. Both ions correspond to the $[M-HF]^-$ fragment from the molecular ion of respectively the nondeuterated and the tetradeuterated *N*-(2-hydroxyethyl) valine-pentafluorophenylthiohydantoin.

3. *Urine Samples*

The urine samples can be stored at −80°C for 6 months. Cotinine quantification was performed by the gas-chromatographic (GC) technique. Preparation of samples and calibration curve and GC analysis have been described in a previous study (Bono *et al.*, 1996).

4. *Statistical Analysis*

All analyses were performed with statistical analysis system packages (SAS/STAT 1992).

C. Quality Controls and Preparatory Results

(1) Questionnaire. The questionnaire was readministered to 40 subjects after one month by the same interviewer. Agreement between replies to the same 15 questions corresponded to *K* indices ranging from 0.83 to 0.94, where *K* (index of Cohen) = (observed agreements − expected agreements) × (1 − expected agreements)$^{-1}$.

(2) Calibration curve for HOEtVal. The degree of purity of the synthesized racemic HOEtVal was approximately 95%. By chromatographic comparison to this HOEtVal, the degrees of alkylation of a background standard globin alkylated *in vitro* with EtO and [$^2H_4$]EtO were calculated and findings obtained were calibration standard (EtO) = 6.3 μmol/gr of globin; internal standard [$^2H_4$]EtO = 5.35 μmol/g of globin. From these results preparation and analysis of calibration curve were made possible. The detection limit calculated by several dilutions of a "background" sample was 10 pmol of HOEtVal/gr of Hb. Coefficients of variation (CV%) of the reproducibility concerning the internal standard was 6.2%, while for samples was 3.5%. Figure 3 shows the calibration curve obtained and the corresponding correlation coefficient (r^2).

(3) Quality control of urinary cotinine was described in a previous publication (Bono *et al.*, 1996).

RESULTS

Table 1 reports the number of subjects involved in the study, by gender and age. A descriptive analysis of *N*-(2-hydroxyethyl) valine and urinary cotinine is reported in Table 2, considering the whole population and three type of exposures to tobacco smoke. *A priori* data regarding the two biological markers were split into three categories, according to smoking habits declared in the questionnaires, namely nonsmokers, passive smokers (living or working with someone who smokes), and active smokers. The increase of exposure intensity to tobacco smoke directly correlates with the increase of concentration for both biological markers. Figures 4 and 5 show the linear regressions between the number of cigarettes smoked by all 146 subjects and HOEtVal and urinary cotinine, respectively. Showing high correlation coefficients and significance, the two statistical analyses demonstrate a close relationship between exposure to cigarette smoke and the two selected biological markers. The linear regression directly calculated between the two markers is shown in Fig. 6. Also, in this case, a high direct relationship is statistically demonstrated, considering the correlation coefficient and significance. This finding confirms the hypothesis that cotinine, an internal dose marker specific for tobacco smoke, can show that the formation of HOEtVal, an aspecific biological effective dose marker, depends also on the exposure to ethene and EtO present in tobacco smoke. Therefore, cotinine level determination can be used indirectly to point out early biological effects due to tobacco

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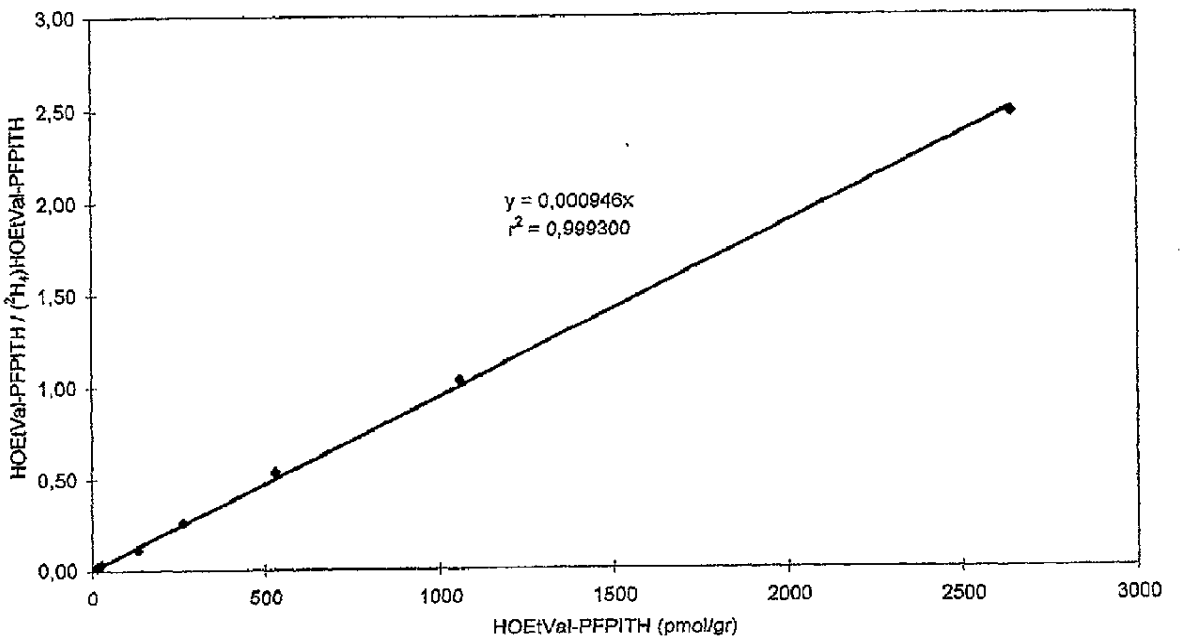

FIG. 3. Calibration curve of HOEtVal.

smoke exposure. At the bottom of Fig. 6, the relationship between the two biological markers is reported by means of the coefficients of correlation and significance, considering the three subgroup of exposure. This relationship is very close for active smokers; however, HOEtVal and cotinine in the other two subgroups of population are also significantly correlated.

At the top of Fig. 7 the means and 95% confidence intervals of HOEtVal in the three main groups of exposure to tobacco smoke are reported. At the bottom of the same figure results relative to ANOVA analysis are shown. The general model is very significant ($P = 0.0001$), confirming the different distribution of the three groups of concentrations. In particular, taking the nonsmokers as the reference category, the entire distribution difference evidenced in the model can be attributed to the group of active smokers. Analogous graphical representation of data and similar results are found (Fig. 8) for urinary cotinine.

Last, Table 3 shows results distributed by sex. The results obtained from *t*-test analysis demonstrate, for both biological markers, higher mean levels for

TABLE 1
Numerical Analysis of Subjects by Gender and Age

No. of subjects	146
Male	82
Female	64
Age (SD)	36.3 (12.63)

TABLE 2
Descriptive Analysis of *N*-(2-Hydroxyethyl)valine (HOEtVal) and Cotinine

	HOEtVal (pmol/g Hb)	Cotinine (ng/ml)
Whole population		
Number	146	146
Mean (SD)	29.7 (39.20)	158.25 (476.21)
Median ($Q_3 - Q_1$)	13.07 (30.81)	16.11 (35.4)
Non smokers		
Number	74	74
Mean (SD)	16.97 (21.05)	16.00 (17.66)
Median ($Q_3 - Q_1$)	9.09 (9.97)	11.6 (12.5)
Passive smokers		
Number	28	28
Mean (SD)	16.58 (23.32)	16.62 (11.85)
Median ($Q_3 - Q_1$)	10.74 (6.53)	15 (10.45)
Active smokers		
Number	44	44
Mean (SD)	59.47 (52.83)	487.60 (777.95)
Median ($Q_3 - Q_1$)	45.43 (38.29)	124.7 (673.9)

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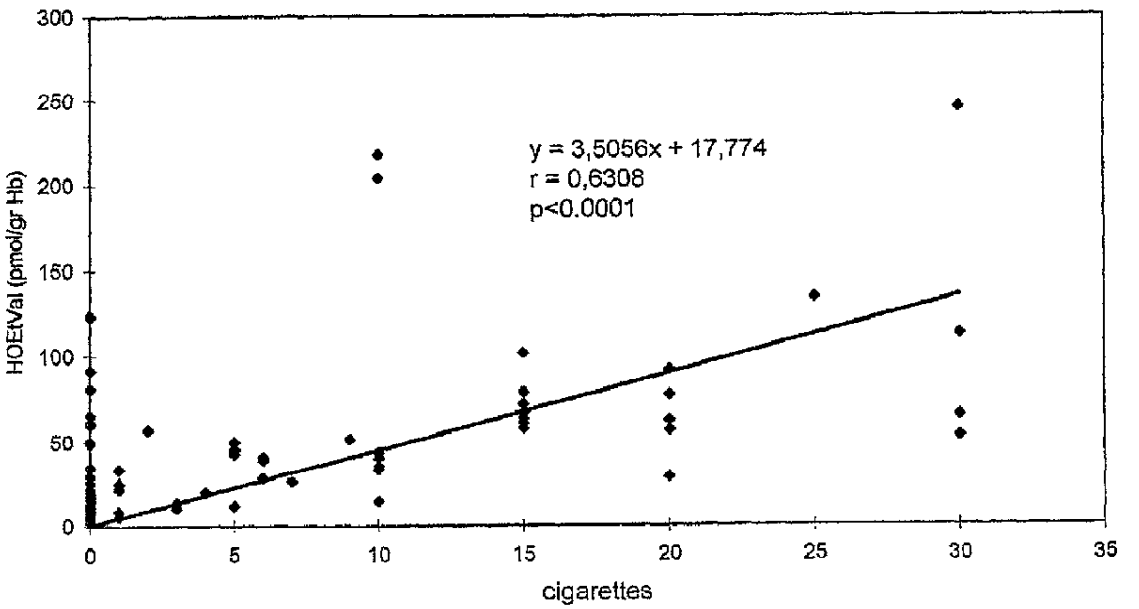

FIG. 4. Linear regression between HOEtVal and number of cigarettes of the whole population.

males. Partial yet nonexhaustive contribution to this unexpected finding can be found in Table 4, showing that males smoke on average a higher number of cigarettes both actively and passively.

DISCUSSION

The procedure adopted to determine HOEtVal demonstrated good sensitivity and reproducibility.

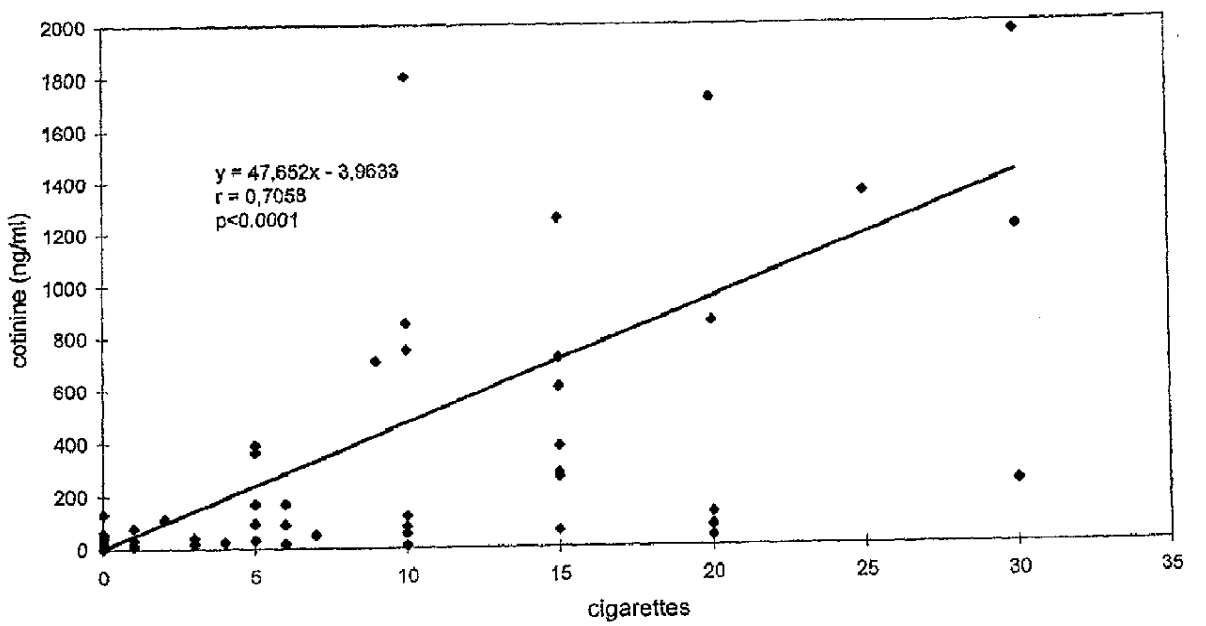

FIG. 5. Linear regression between urinary cotinine and number of cigarettes of the whole population.

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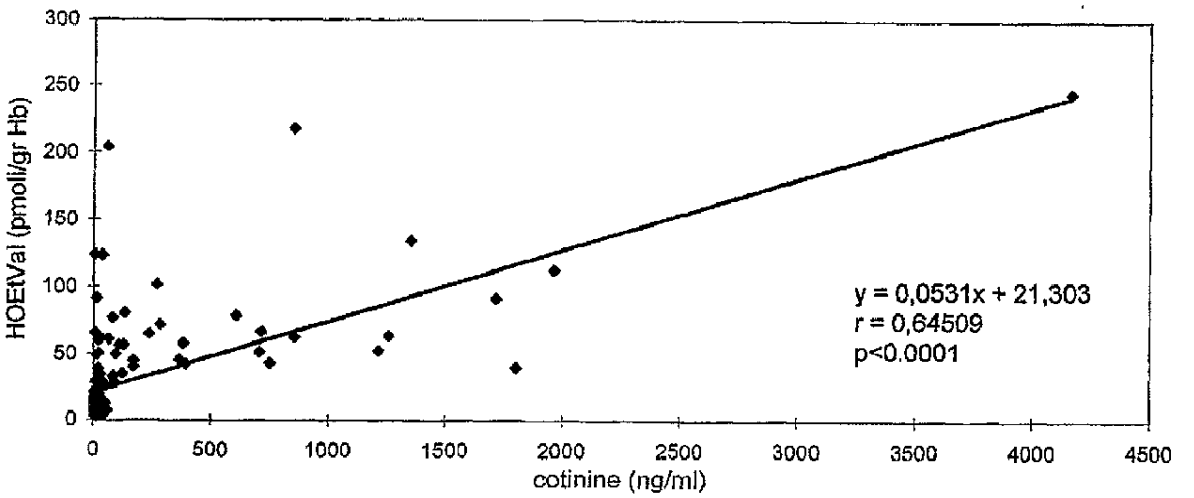

HOEtVal v.s. cotinine

	r	p
NON SMOKERS	0.23096	0,0477
PASSIVE SMOKERS	0.38938	0,0405
ACTIVE SMOKERS	0.63364	0.0001

FIG. 6. Linear regression between urinary cotinine and HOEtVal of the whole population. Coefficient of correlatiton and significance of the three population subgrouped by smoking habit are also reported.

The optimal calibration curve obtained proved that each phase of this analysis can be considered properly carried out.

From this study, tobacco smoke intake stands out as a prominent factor in the epidemiological distribution of HOEtVal within the selected group of urbanized healthy subjects. Whenever work-related and endogenous sources of HOEtVal can be excluded and taking into account that the environmental air exposure to EtO in Turin city is negligible, HOEtVal distribution in hemoglobin shows a direct positive relationship to tobacco smoke exposure. Further support to this conclusion arises from findings relative to urinary cotinine, which confirm a very close correlation with HOEtVal level and with the number of cigarettes actively or passively smoked, as defined from subjective declaration.

The two biological markers represent two very important phases of the process connecting exposure to tobacco smoke with the risk of tumor onset. The first step, exposure, takes into account that the urinary cotinine level plays the role of internal *dose* marker, effectively detecting (total specificity and very high sensitivity) the magnitude of tobacco smoke exposure. The HOEtVal, a molecular adduct

TABLE 3

Gender Distribution of HOEtVal and Cotinine Values

	HOEtVal (pmol/g Hb)	Cotinine (ng/ml)
Males		
Number	82	82
Mean (SD)	40.92 (47.94)	234.90 (604.75)
Median ($Q_3 - Q_1$)	21.01 (47.79)	23.9 (67.9)
Females		
Number	64	64
Mean (SD)	15.34 (14.44)	60.04 (185.14)
Median ($Q_3 - Q_1$)	10.08 (9.14)	12.85 (12.95)
t-test	male vs female ($p = 0.00001$)	male vs female ($p = 0.00001$)

TABLE 4

Cigarettes/Actively and Passively Smoked by Sex

	Male (*N*)	Female (*N*)
Cigarettes/day actively smoked	12.4 (30)	8.9 (14)
Cigarettes/day passively smoked	18.5 (16)	12.0 (12)

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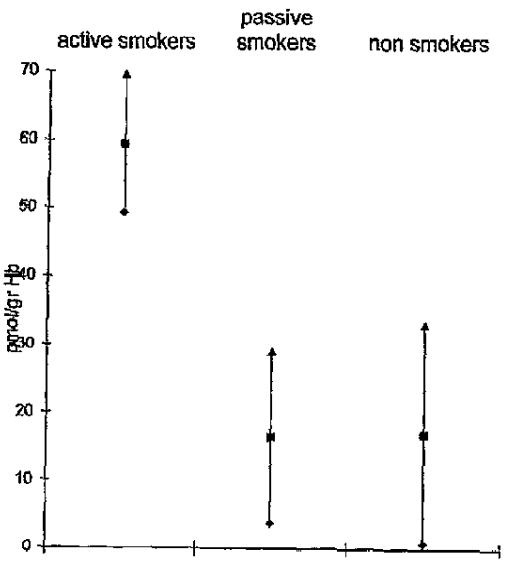
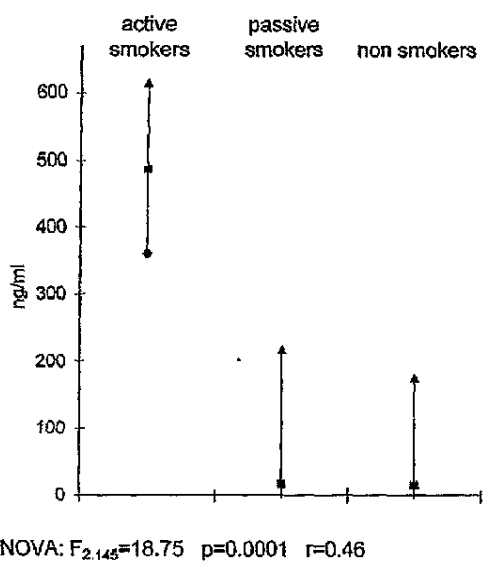


ANOVA: $F_{2,145}$=23.877 p=0.0001 r=0.50

COMPARISONS	HOEtVal
non smokers v.s. passive smokers	n.s.
non smokers v.s. active smokers	<0.05
passive smokers v.s. active smokers	<0.05

FIG. 7. Distribution of HOEtVal (means and 95% confidence intervals) in the three subgroup. Findings of ANOVA analysis are also reported.



ANOVA: $F_{2,145}$=18.75 p=0.0001 r=0.46

COMPARISONS	cotinine
non smokers v.s. passive smokers	n.s.
non smokers v.s. active smokers	<0.05
passive smokers v.s. active smokers	<0.05

FIG. 8. Distribution of urinary cotinine (means and 95% confidence intervals) in the three subgroup. Findings of ANOVA analysis are also reported.

to a protein, shows an early biological *effect* that indirectly reflects, in proportional way, early damage to DNA and, consequently, the possible onset of a carcinogenic process.

Results relative to both HOEtVal and urinary cotinine in adults do not allow distinction between passive smokers and nonsmoker groups or distinctions inside the group of passive smokers. Nevertheless, several authors (Tredaniel *et al.* 1994; Bono *et al.*, 1996) demonstrated that cotinine has a very good capacity for distinction within the passsive smoker group. In particular, in the above-mentioned study we showed that urinary cotinine measured in a large group of adolescents can well discriminate different degrees of passive exposure to tobacco smoke declared by questionnaire (Bono *et al.*, 1996). It is possible that this reflects the simpler life style of the adolescents (14 years old) who were nonsmokers almost exclusively, but were exposed to tobacco smoke of their parents or other cohabitants.

The more complex life style of the adult poupulation expands the chance of passive exposure to tobacco smoke (at home, at work, during free time, etc.) and, consequently, minimizes the difference between urinary cotinine in nonsmokers and in passive smokers. The same lack of difference in HOEtVal levels is observed, as expected.

The future objective of the present study is a closer inspection of the two biomarkers with respect to passive exposure to tobacco smoke. To achieve this objective and to verify the sensitivity of the HOEtVal marker, we intend to:

- complete the present study involving a larger number of adult subjects
- carry out a new epidemiological study considering a large group of adolescents
- increase the sensitivity of GC/MS analysis using tandem mass spectrometric techniques with the aim of lowering the detection limit to 1 pmol/g.

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